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Transcriptional upregulation of both *egl-1* BH3-only and *ced-3* caspase is required for the death of the male-specific CEM neurons

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Summary

Most of the 131 cells that die during the development of a *C. elegans* hermaphrodite do so ~30 min after being generated. Furthermore, in these cells, the pro-caspase proCED-3 is inherited from progenitors and the transcriptional upregulation of the BH3-only gene *egl-1* is thought to be sufficient for apoptosis induction. In contrast, the four CEM neurons, which die in hermaphrodites, but not males, die ~150 min after being generated. We found that in the CEMs, the transcriptional activation of both the *egl-1* and *ced-3* gene is necessary for apoptosis induction. In addition, we show that the Bar homeodomain transcription factor CEH-30 represses *egl-1* and *ced-3* transcription in the CEMs, thereby permitting their survival. Furthermore, we identified three genes, *unc-86*, *lrs-1* and *unc-132*, which encode a POU homeodomain transcription factor, a leucyl-tRNA synthetase and a novel protein with limited sequence similarity to the mammalian proto-oncoprotein and kinase PIM-1, respectively, that promote the expression of the *ceh-30* gene in the CEMs. Based on these results, we propose that *egl-1* and *ced-3* transcription are co-regulated in the CEMs to compensate for limiting proCED-3 levels, which most probably are a result of proCED-3 turn over. Similar co-regulatory mechanisms for BH3-only proteins and pro-caspases may function in higher organisms to allow efficient apoptosis induction during development. Finally, we present evidence that the timing of the death of the CEMs is controlled by TRA-1 Gli, the terminal global regulator of somatic sexual fate in *C. elegans*.

Introduction

The elimination of unwanted cells by apoptosis is critical for animal development (1). A central enzymatic machinery involved in the induction of apoptosis has been defined, and found to be conserved in animals as diverse as the nematode *Caenorhabditis elegans* and

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humans (2–5). It is generally believed that most if not all cells in developing animals contain this machinery in an inactive state. Furthermore, BH3-only proteins, which function as receivers of apoptotic stimuli, have emerged as key activators of this central cell-death machinery in both *C. elegans* and vertebrates (6, 7). However, many questions about apoptosis induction remain to be answered. For example, it is unclear how animals ensure the presence of the central cell death machinery in most if not all cells during development, which is a prerequisite for BH3-only-dependent apoptosis induction.

Due to its invariant pattern of apoptotic cell death during development, *C. elegans* has become an important model for apoptosis studies. The central cell death machinery in *C. elegans* is composed of the anti-apoptotic BCL-2-like protein CED-9, the APAF-1-like caspase activator CED-4 and the pro-caspase proCED-3 (4, 5, 8). At least during mid-embryogenesis when the majority of cell deaths occurs, most if not all cells in developing embryos contain CED-4 as well as proCED-3, which once matured is sufficient for apoptosis induction (9–11). However, these cells also contain the CED-9 protein, which through its direct interaction with CED-4, blocks the ability of CED-4 to mediate proCED-3 maturation (9, 10). As a result, apoptosis induction is prevented. During the development of a *C. elegans* hermaphrodite, exactly 131 cells are programmed to die (12). The majority of these cells die ~30 min after being generated. Furthermore, in these cells, the transcriptional activation of the BH3-only gene *egl-1* is thought to be sufficient for the activation of the central cell death machinery and, hence, apoptosis induction (13–16). Specifically, the BH3-only protein EGL-1 can bind to CED-9 thereby causing CED-4 release from CED-9 and CED-4-dependent proCED-3 maturation. Interestingly, recent data suggest that the *ced-3* gene is not transcriptionally active in most of the 131 cells that are programmed to die during development (10). This observation suggests that proCED-3 protein present in these cells is derived from their progenitors. It also suggests that once activated, the amount of proCED-3 protein inherited from progenitors is, in general, sufficient for apoptosis induction.

The four male-specific cephalic companion neurons (CEMs) are generated ~320 min after the first cell division. In males, the CEMs survive and differentiate into ciliated sensory neurons implicated in mating behavior (12, 17, 18). In hermaphrodites, however, the CEMs undergo apoptosis ~150 min after being generated (~470 min after the first cell division). As is the case for most of the cells that are programmed to die during development, the death of the CEMs is dependent on the genes *egl-1*, *ced-4* and *ced-3* (13, 19). However, the death of the CEMs is also dependent on the gene *tra-1*, which encodes the terminal, global regulator of somatic sexual fate and which acts to promote female development (20). Specifically, the TRA-1 protein, a GLI-like transcription factor, causes the CEMs in hermaphrodites to undergo apoptosis by directly repressing the transcription of the gene *ceh-30*, which encodes a Bar homeodomain transcription factor that acts to block the death of the CEMs in males (21, 22).

How the CEH-30 protein acts on the central cell death machinery to block the death of the CEMs has so far been unclear. We now present evidence that CEH-30 blocks the death of the CEMs by repressing the transcription of both the *egl-1* and *ced-3* gene. Furthermore, we identified three genes, *unc-86*, *lrs-1*, and *unc-132*, which encode a POU homeodomain

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transcription factor, a leucyl-tRNA synthetase and a novel protein with limited sequence similarity to the mammalian proto-oncoprotein and kinase PIM-1, respectively, that promote *ceh-30* expression in the CEMs thereby antagonizing *tra-1* function. Our data also demonstrate that in contrast to most cells that are programmed to die during development, in the CEMs, the transcriptional upregulation of both the BH3-only gene *egl-1* and the caspase gene *ced-3* is required for apoptosis induction. We propose that coupling the transcriptional upregulation of a key activator of apoptosis induction (*egl-1*) and a component of the central, enzymatic cell death machinery (*ced-3*) ensures efficient apoptosis induction in the CEMs.

Results

bc151, *bc155*, and *bc159* cause differentiated CEMs to be absent from masculinized hermaphrodites or males

An altered-function mutation of the *C. elegans* gene *sel-10*, *n1077*, which encodes an F-box protein that promotes female development, causes weak masculinization of the hermaphrodite soma (23–25). For example, in wild-type hermaphrodites, the four CEMs undergo apoptosis (12, 17). In contrast, in *sel-10(n1077)* hermaphrodites, the CEMs inappropriately survive. The CEMs in *sel-10(n1077)* hermaphrodites have the characteristic CEM morphology when observed by differential interference contrast microscopy (DIC) in larvae of the fourth larval stage (L4 larvae), and express CEM-specific markers in adults, such as the genes *pkd-2* ($P_{pkd\text{-}2}gfp$) and *lov-1* ($P_{lov\text{-}1}gfp$) (see below) (26). These observations indicate that the CEMs in *sel-10(n1077)* hermaphrodites are correctly specified and fully differentiated.

To identify genes required for the survival of the CEMs, we sought mutations that cause the CEMs to be absent from *sel-10(n1077)* hermaphrodites based on the expression of *pkd-2*. Using this criterion, we screened 8,500 mutagenized, haploid genomes and identified 49 mutants. Among these mutants, 21 still had CEMs based on DIC and therefore most likely harbor mutations that affect *pkd-2* expression. In addition, in 20 mutants, all aspects of the weak masculinization caused by *sel-10(n1077)* were suppressed, indicating that they most likely carry feminizing mutations or loss-of-function mutations in the *sel-10* gene. Of the remaining eight mutations, one behaved non-Mendelian, one could not be outcrossed, and three caused the CEMs to be absent in masculinized *sel-10(n1077)* hermaphrodites, but not males. The final three mutations, *bc151*, *bc155*, and *bc159*, were characterized further.

Based on *pkd-2* expression in adults, the recessive mutations *bc151*, *bc155*, and *bc159* cause the majority of CEMs to be absent from masculinized hermaphrodites as well as males (Table 1A; data not shown). The CEMs are also absent in *bc151*, *bc155*, or *bc159* masculinized hermaphrodites based on their characteristic morphology in L4 larvae (Table 1B) and in *bc151*, *bc155*, or *bc159* males based on *lov-1* expression (Table S1). In addition, all three mutations cause a reduced brood size and an uncoordinated or ‘Unc’ phenotype (Table S2; data not shown). None of the mutations affects viability at least in the *sel-10(n1077)* background (Table S3). Based on these observations, we conclude that *bc151*, *bc155*, and *bc159* cause differentiated CEMs to be absent in masculinized hermaphrodites and males.

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bc151 is a loss-of-function mutation of *unc-86* POU

unc-86, which encodes a POU transcription factor, has previously been shown to be required for the presence of differentiated CEMs in males (21, 27, 28). We found that *bc151* animals carry two mutations in the *unc-86* gene: first, a missense mutation leading to an aspartic acid (GAU) -to-asparagine (AAU) substitution at position 203 of the protein sequence of the UNC-86.b protein (generated by transcript *C30A5.7b*); second, a nonsense mutation, changing codon 220 of the *C30A5.7b* coding sequence (CAA) to a STOP codon (UAA) (Figure 1A). Both *unc-86* transcripts (*C30A5.7a*, *C30A5.7b*) are predicted to be affected by the two mutations. Since loss-of-function mutations of *unc-86* have previously been shown to cause differentiated CEMs to be absent in males, we conclude that *bc151* is a new loss-of-function mutation of the *unc-86* gene.

bc155 is a loss-of-function mutation of the gene *lrs-1* leucyl-tRNA synthetase

We found that *bc155* is a mutation in the gene *lrs-1*, which encodes a leucyl-tRNA synthetase, a Class I tRNA synthetase (Figure 1B; data not shown) (29). *bc155* is a G-to-A transition that changes the 3' splice acceptor site of intron 3 from AG to AA, which results in reduced levels of fully spliced *lrs-1* mRNA (Figure 1B; data not shown). Both *lrs-1* transcripts (*R74.1.1*, *R74.1.2*) are predicted to be affected by the *bc155* mutation. Reducing *lrs-1* function by RNAi results in the absence of differentiated CEMs in masculinized hermaphrodites (Table 2A). Based on these findings, we conclude that *bc155* is a loss-of-function mutation of the gene *lrs-1*. Furthermore, we found that reducing the function by RNAi of genes encoding other Class I tRNA synthetases (isoleucyl-tRNA synthetase [*irs-2*], valyl-tRNA synthetase [*vrs-2*]) or Class II tRNA synthetases (histidyl-tRNA synthetase [*hrs-1*], tryptophanyl-tRNA synthetase [*wrs-2*], threonyl-tRNA synthetase [*trs-1*]) can result in the absence of at least 20% of differentiated CEMs in masculinized hermaphrodites (Table 2A). Hence, the presence of differentiated CEMs in masculinized hermaphrodites is sensitive to changes in the activity level of the translational machinery.

bc159 is a loss-of-function mutation of the PIM-1-like gene *unc-132*

bc159 is a mutation in a previously uncharacterized gene, *W08A12.1*, which generates five transcripts. Transcript *W08A12.1.a* encodes a 501 amino acid protein of unknown function with a P-loop domain and sequence similarity to an uncharacterized protein of *Drosophila melanogaster*, CG30118, and the human proto-oncoprotein and kinase PIM-1 (Figure 1C; see below) (30, 31). As mentioned above, apart from causing differentiated CEMs to be absent in masculinized hermaphrodites and males, *bc159* also causes an Unc phenotype. For this reason, we named *W08A12.1* '*unc-132*'. The *bc159* mutation affects the second exon that is common to all five *unc-132* transcripts. Specifically, *bc159* is a missense mutation that leads to a methionine (AUG) -to-isoleucine (AUA) substitution at position 200 of the protein sequence of the UNC-132.a protein (generated by transcript *W08A12.1.a*). Since the reduction of *unc-132* function by RNAi causes differentiated CEMs to be absent in a *sel-10(n1077)* background (Table 2B), we conclude that *bc159* is a loss-of-function mutation of the *unc-132* gene.

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unc-132 is orthologous to the gene *CG30118* of *D. melanogaster* (30). Over its entire length, the UNC-132 protein is 38.2% identical and 53.5% similar to the CG30118 protein (Figure 1D). The similarities between UNC-132 and CG30118 are mainly found in the central (57.5% identity, 76.4% similarity) and C-terminal (42.3% identity, 59.6% similarity) regions of the two proteins. In addition, both proteins contain a P-loop domain. Among mammalian proteins, UNC-132 is most similar to the human kinase PIM-1 (12.7% identical and 22.0% similar over its entire length); however the two proteins are not orthologous (31). The similarity between UNC-132 and PIM-1 is mainly restricted to their N-terminal (29.9% identical, 46.8% similar) regions. As is the case for UNC-132 and CG30118, PIM-1 has a P-loop domain. However, in contrast to UNC-132 and CG30118, PIM-1 also contains a kinase domain.

The loss of *unc-86*, *lrs-1* or *unc-132* function causes a defect in CEM specification

To determine whether the CEMs inappropriately undergo apoptosis in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function, we tested whether a mutation that blocks apoptotic cell death in general (*ced-3*(*n717*)) can suppress the CEM phenotype observed in these mutant backgrounds (19). We found that *ced-3*(*n717*) failed to suppress the lack of differentiated CEMs in these animals, which indicates that the loss of *unc-86*, *lrs-1* or *unc-132* function does not cause the CEMs to inappropriately die (Table 1A, B). This notion is supported by the following observation. In wild-type hermaphrodites, cells with a refractile, corpse-like morphology indicative of apoptotic cells are detected at the position at which the CEMs are located ~470 min after the first cell division, which is the time at which the CEMs die in these animals (Table S4). In contrast, in masculinized hermaphrodites, in which the CEMs survive, such cells are not detected. Similarly, in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function, cells with a refractile, corpse-like morphology are rarely detected at this position (Table S4).

To determine whether the CEMs are generated, we analyzed the fate of their sisters, the dorsal URAs in the case of the dorsal CEMs and the amphid socket cells in the case of the ventral CEMs (12). The rationale for these analyses was that the presence of properly differentiated sisters would suggest that the cell division that generates the CEMs and their sisters had occurred appropriately. Using markers specifically expressed in differentiated URAs ($P_{glr\text{-}4}$*glr-4::gfp* and $P_{cfi\text{-}1}$*gfp*) or amphid socket cells ($P_{unc\text{-}53}$*gfp*) (32–34), we found that the loss of *lrs-1* or *unc-132* function does not affect the presence of differentiated dorsal URAs or amphid socket cells (Table 3). Based on these results we conclude that the CEMs are generated in animals lacking *lrs-1* or *unc-132* function. In the case of *unc-86*, we found that animals lacking *unc-86* function had amphid socket cells, but lacked differentiated dorsal URAs (Table 3). However, as shown below, we can detect CEMs in *unc-86* mutant embryos using a transcriptional *egl-1* reporter. Therefore, we conclude that the CEMs are also generated in animals lacking *unc-86* function. Based on these observations we propose that the loss of *unc-86*, *lrs-1* or *unc-132* function causes a defect in the specification (rather than generation or survival) of the CEMs.

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unc-86, *lrs-1* and *unc-132* are required for *ceh-30* expression in the CEMs in masculinized hermaphrodites

The Bar homeodomain transcription factor CEH-30, whose expression is under the direct control of the terminal, global regulator of somatic sexual fate, TRA-1, acts to block the death of the CEMs (21, 22). Specifically, the loss of *ceh-30* function causes the CEMs to inappropriately die in males as well as in masculinized hermaphrodites, indicating that *ceh-30* is required for CEM survival (21, 22) (Grote, P. and Conradt, B., unpublished data). Using a translational *ceh-30* reporter ($P_{ceh\text{-}30}$*ceh-30::gfp*) (22), we determined the expression pattern of the *ceh-30* gene in the CEMs in embryos ~450 min after the first cell division, which is just prior to the time at which the CEMs normally die in hermaphrodites (~470 min) (12, 16). The CEMs were identified based on their position using DIC and observed for ~30 min. We found that in wild-type hermaphrodites, the CEMs do not express *ceh-30* and adopt a corpse-like morphology (Figure 2A, B, +/+). In contrast, in males (XO +/+) or masculinized hermaphrodites (*sel-10*(*n1077*)), the CEMs do express *ceh-30* and do not adopt a corpse-like morphology (Figure 2A, B). These observations confirm that *ceh-30* expression correlates with CEM survival. These observations also confirm that inappropriately surviving CEMs in masculinized hermaphrodites faithfully recapitulate molecular events that occur in the CEMs in males.

Next we analyzed the expression of the *ceh-30* reporter in the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function in which, as described above, the CEMs are generated and survive, but are mis-specified. We found that in these animals, *ceh-30* is not expressed in most CEMs (Figure 2A, B). This result was surprising since the loss of *ceh-30* function causes the CEMs to inappropriately die in masculinized hermaphrodites. Based on these observations we conclude that *unc-86*, *lrs-1* and *unc-132* are required for the expression of *ceh-30* in the CEMs in masculinized hermaphrodites (Figure 5). We also conclude that apart from promoting *ceh-30* expression, *unc-86*, *lrs-1* and *unc-132* have an additional function, which is to promote CEM death in a *ceh-30*-independent manner (Figure 5).

The loss of *unc-86*, *lrs-1* or *unc-132* function causes de-repression of *egl-1* transcription in the CEMs in masculinized hermaphrodites

Using a transcriptional reporter ($P_{egl\text{-}1}$*his-24::gfp*), we analyzed the expression of the *egl-1* gene in the CEMs ~450 min after the first cell division (12, 16). We found that in wild-type hermaphrodites, the CEMs transcribe *egl-1* and adopt a corpse-like morphology (Figure 3A, B, +/+). In contrast, in masculinized hermaphrodites, the CEMs do not transcribe *egl-1* and do not adopt a corpse-like morphology (Figure 3A, B, *sel-10*(*n1077*)). As mentioned above, the loss of *ceh-30* function causes the CEMs to inappropriately die in masculinized hermaphrodites. We found that the loss of *ceh-30* function causes *egl-1* to be inappropriately transcribed in the CEMs in masculinized hermaphrodites (Figure 2B, C, *ceh-30*(*bc272*); *sel-10*(*n1077*)). These results demonstrate that *egl-1* transcriptional upregulation in the CEMs correlates with CEM death. In addition, they demonstrate that *ceh-30* functions to repress *egl-1* transcription in the CEMs in masculinized hermaphrodites (Figure 5).

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To determine why the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function, but not *ceh-30* function, fail to inappropriately die, we analyzed *egl-1* transcription in the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132*. To our surprise, we found that the CEMs in these animals transcribe *egl-1* (Figure 3A, B). This observation indicates that, unlike in masculinized hermaphrodites lacking *ceh-30* function, in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function, the transcriptional upregulation of *egl-1* in the CEMs is not sufficient for their apoptotic death.

ced-3 is transcribed in the CEMs in hermaphrodites and its transcription in the CEMs in masculinized hermaphrodites is repressed by *ceh-30*

Using a reporter for the caspase gene *ced-3*, it has been shown that *ced-3* is not transcribed in most of the 131 cells that are programmed to die (10). However, *ced-3* is actively transcribed in at least one cell that is programmed to die, the tail spike cell (10). Like the CEMs, the tail spike cell survives much longer than most cells programmed to die (~300 min) before it dies. For this reason, we explored whether the *ced-3* gene is transcribed in the CEMs prior to their deaths in hermaphrodites.

Using a transcriptional reporter ($P_{ced\text{-}3}gfp$), we found that *ced-3* is transcribed in most CEMs in wild-type hermaphrodites, in which the CEMs die (Figure 4 A, B, +/+). Therefore, the *ced-3* gene is transcriptionally active in the CEMs. Furthermore, we found that in masculinized hermaphrodites, in which the CEMs survive, *ced-3* is not transcribed (Figure 4 A, B, *sel-10*(*n1077*)). These observations are consistent with the notion that, like *egl-1* transcription, the transcription of *ced-3* in the CEMs correlates with CEM death. Next we determined the role of *ceh-30* in *ced-3* transcription in the CEMs. We found that in masculinized hermaphrodites lacking *ceh-30* function, in which the CEMs inappropriately die, *ced-3* was expressed in the CEMs (Figure 4 A, B). Therefore, we conclude that *ceh-30* functions to repress *ced-3* transcription in the CEMs in masculinized hermaphrodites (Figure 5).

The loss of *unc-86*, *lrs-1* or *unc-132* function does not cause de-repression of *ced-3* transcription in the CEMs in masculinized hermaphrodites

To determine the role of *unc-86*, *lrs-1* and *unc-132* in *ced-3* transcriptional control in the CEMs, we analyzed the expression of the $P_{ced\text{-}3}gfp$ reporter in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function. We found that, like in masculinized hermaphrodites, the $P_{ced\text{-}3}gfp$ reporter was not expressed in most CEMs (Figure 4 A, B). Therefore, while the loss of *unc-86*, *lrs-1* or *unc-132* function results in the de-repression of *egl-1* transcription in the CEMs in masculinized hermaphrodites, it does not result in the de-repression of *ced-3* transcription. Based on this observation we conclude that *unc-86*, *lrs-1* and *unc-132* not only repress *ced-3* transcription in a *ceh-30*-dependent manner but also promote *ced-3* transcription in a *ceh-30*-independent manner (Figure 5). Furthermore, we propose that the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1* or *unc-132* function fail to die because *ced-3* transcription in the CEMs is not de-repressed.

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ceh-30 expression inversely correlates with *egl-1* and *ced-3* transcription

Our results indicate that the death of the CEMs in wild-type hermaphrodites is the result of TRA-1-dependent repression of *ceh-30* transcription, which results in the de-repression of *egl-1* and *ced-3* transcription in the CEMs. To test this hypothesis, we monitored *ceh-30* expression as well as *egl-1* and *ced-3* transcription throughout the life span of the CEMs in hermaphrodites using 4D lineaging analysis and appropriate reporters ($P_{ceh\text{-}30}$*ceh-30::gfp*, $P_{egl\text{-}1}$*his-24::gfp*, $P_{ced\text{-}3}$*gfp*). We found that *ceh-30* expression in the CEMs in wild-type hermaphrodites can only be detected right after the cells are generated (~340 min) (Figure 6, Figure S1). *ceh-30* expression cannot be detected in the mother cell or in the CEMs at ~360 min or later time points. In contrast, *egl-1* and *ced-3* transcription can only be detected starting at ~390 and ~460 min, respectively (Figure 6, Figure S1). These observations demonstrate that *ceh-30* expression in the CEMs inversely correlates with *egl-1* and *ced-3* transcription, which supports the model that TRA-1-dependent repression of *ceh-30* transcription causes the de-repression of *egl-1* and *ced-3* transcription in the CEMs in wild-type hermaphrodites and, hence, CEM death.

Discussion

Transcriptional upregulation of both *egl-1* and *ced-3* is required for the apoptotic death of the CEMs in hermaphrodites

The transcriptional upregulation of *egl-1* is thought to be necessary and sufficient for many of the cell death events that take place during development (14–16). We found that *egl-1* transcriptional upregulation also correlates with cell death in the CEMs. Specifically, *egl-1* is transcriptionally active in the CEMs in hermaphrodites in which the CEMs die but not in masculinized hermaphrodites (and most probably males) in which the CEMs survive. However, our results demonstrate that the transcriptional upregulation of *egl-1* in the CEMs in hermaphrodites is necessary, but not sufficient for their death.

The *ced-3* gene does not appear to be transcriptionally active in many of the cells that are programmed to die during development (10). However, we found that *ced-3* is transcriptionally active in the CEMs in hermaphrodites in which the CEMs die but not in masculinized hermaphrodites in which the CEMs survive. Furthermore, we present data that indicate that transcriptional activation of *ced-3* in the CEMs is necessary for their death in hermaphrodites. Based on these findings we propose that in contrast to most cells that are programmed to die during development, in the CEMs, the transcriptional upregulation of *egl-1* and *ced-3* is necessary for their death in hermaphrodites. At least to our knowledge, this is the first demonstration that the transcriptional upregulation of both a BH3-only gene as well as a caspase gene is required for apoptosis induction.

Why would the transcriptional upregulation of not only *egl-1* but *ced-3* be necessary for apoptosis induction in the CEMs? What distinguishes the CEMs from the majority of cells that die during development is that rather than surviving for only ~30 min after being generated, they survive for ~150 min (12). In the majority of cells that are programmed to die proCED-3 protein is inherited from progenitors (10). Based on this observation, we propose that at the time apoptosis induction takes place in the CEMs, as a result of

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proCED-3 turn over, the level of progenitor-derived proCED-3 has reached a level in the CEMs that is no longer sufficient for apoptosis induction (Figure 6A). For this reason, the transcriptional upregulation of not only the *egl-1* gene but also the *ced-3* gene is necessary for apoptosis induction in the CEMs.

Our model is supported by findings on the death of the tail spike cell. The tail spike cell, which may play a role in the morphogenesis of the tail, does not die until ~300 min after being generated (12). As is the case for the majority of cell death events as well as the death of the CEMs, the death of the tail spike cell is absolutely dependent on *ced-3* (10). However, in contrast to the majority of cell death events as well as the death of the CEMs, it is only partially dependent on *egl-1* (10). In addition, as in the CEMs, the *ced-3* gene is transcriptionally active in the tail spike cell prior to its death. Furthermore, mutations in the gene *pal-1*, which encodes a caudal-like transcription factor, can prevent *ced-3* transcriptional activation in the tail spike cell and tail spike cell death (10). What is unclear at the moment is whether *egl-1* is also transcriptionally activated in the tail spike cell prior to its death. The over-expression of the *ced-3* gene can be sufficient for apoptosis induction in the absence of apoptotic stimuli and *egl-1* transcriptional upregulation (i.e. in cells programmed to live) (11). Therefore, we speculate that in the tail spike cell, *ced-3* transcription might be upregulated to a level that results in amounts of proCED-3 that can be sufficient for EGL-1-independent apoptosis induction.

unc-86, *lrs-1* and *unc-132* promote *ceh-30* transcription in the CEMs in masculinized hermaphrodites thereby causing repression of *egl-1* and *ced-3* transcription

Loss-of-function mutations of *ceh-30* cause the CEMs to inappropriately undergo apoptosis in males and masculinized hermaphrodites through a yet unknown mechanism (21, 22) (Grote, P. and Conradt, B., unpublished data). The inappropriate death of CEMs in males lacking *ceh-30* function is suppressed by the loss of *egl-1* function (Schwartz and Horvitz, 2007). This suggests that the *egl-1* gene acts downstream of the *ceh-30* gene and is a potential target of the CEH-30 transcription factor. However, analyses performed with a gain-of-function mutation of *ceh-30,* which results in the mis-expression of the CEH-30 protein in the CEMs in hermaphrodites and, consequently, inappropriate survival, suggest that the *ceh-30* gene blocks the death of the CEMs by acting in parallel to or downstream of *egl-1* (22). We present data that indicate that the inappropriate death of CEMs in masculinized hermaphrodites lacking *ceh-30* function is the result of the transcriptional de-repression of the pro-apoptotic genes *egl-1* and *ced-3*. In other words, we show that the *ceh-30* gene acts both upstream of as well as in parallel to *egl-1* to prevent the activation of the central cell death machinery. We propose that CEH-30 is required to repress *egl-1* and *ced-3* transcription in the CEMs of masculinized hermaphrodites, thereby causing their survival (Figure 6B). Whether CEH-30 represses *egl-1* and *ced-3* transcription directly or indirectly remains to be determined.

The *ceh-30* gene is transcriptionally active in the CEMs in males and masculinized hermaphrodites, but not in hermaphrodites. In hermaphrodites, *ceh-30* transcription in the CEMs is directly repressed by the GLI-like transcription factor TRA-1, which is the terminal, global regulator of somatic sexual fate (20–22). Our results indicate that the genes

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unc-86, *lrs-1*, and *unc-132*, which are required for correct CEM specification, are necessary for *ceh-30* transcriptional activation in the CEMs in masculinized hermaphrodites. The sex-specific and cell-specific signals that determine the life-versus-death decision in the CEMs are therefore integrated at the level of *ceh-30* transcriptional control (Figure 5).

unc-86 encodes a POU homeodomain transcription factor that has previously been implicated in CEM specification and differentiation (27, 28). Our results demonstrate that the UNC-86 protein is required for *ceh-30* transcription in the CEMs, which is consistent with previous studies that suggest that UNC-86 acts as a direct activator of *ceh-30* transcription (21). Surprisingly, *lrs-1* encodes a leucyl-tRNA synthetase. Based on our analyses, compromising translation by decreasing the level of at least certain aminoacyl-tRNA synthetases abrogates *ceh-30* transcription in the CEMs. Therefore, we propose that *ceh-30* transcription is particularly sensitive to perturbations of the translational machinery. For example, the translation of factors required for *ceh-30* transcription (such as UNC-86 or UNC-132 or regulators thereof) may be particularly sensitive to such perturbations. Finally, *unc-132* encodes a novel protein of unknown function with a P-loop domain, which suggests that it is capable of nucleotide binding. The *D. melanogaster* orthologue of *unc-132*, *CG30118*, was identified in a screen for genes involved in Drosophila hematopoiesis and is required for the correct localization of crystal cells and plasmatocytes (two classes of terminally differentiated blood cells) in Drosophila embryos (30). However, the mechanism through which *CG30118* affects the localization of these cells has so far not been determined. Furthermore, we found that the N-terminal region of the UNC-132 protein shares sequence similarity with the N-terminal region of the human proto-oncoprotein PIM-1 (31). PIM-1 is a serine/threonine kinase, which is involved in various cellular processes such as cell metabolism, cell proliferation and differentiation and cell survival (35, 36). However, the roles of PIM-1 in these cellular processes are thought to be dependent on its kinase activity. Since UNC-132 does not contain a classical kinase domain, it is therefore unclear how extensive the functional similarities between PIM-1 and UNC-132 are. In summary, while UNC-86 may be a direct activator of *ceh-30* transcription, the mechanisms through which LRS-1 and UNC-132 promote *ceh-30* transcription in the CEMs remain to be elucidated.

unc-86, *lrs-1* and *unc-132* promote *ced-3* transcription in the CEMs of hermaphrodites in a *ceh-30*-independent manner thereby promoting their death

unc-86, *lrs-1*, and *unc-132* are required for *ceh-30* transcriptional activation in the CEMs in masculinized hermaphrodites. However, while we found that the loss of *ceh-30* function results in the de-repression of both *egl-1* and *ced-3* transcription, the loss of *unc-86*, *lrs-1*, or *unc-132* function only results in the de-repression of *egl-1* transcription. For this reason, we propose that apart from blocking *egl-1* and *ced-3* transcription in a *ceh-30*-dependent manner, *unc-86*, *lrs-1*, and *unc-132* also promote *ced-3* transcription in a *ceh-30*-independent manner (Figure 5). Furthermore, we hypothesize that the ability of *unc-86*, *lrs-1*, and *unc-132* to promote *ced-3* transcription in a *ceh-30*-independent manner is relevant in hermaphrodites in which the *ced-3* gene is relieved of *ceh-30*-dependent transcriptional repression. Conversely, their ability to block *ced-3* transcription in a *ceh-30*-dependent manner may be relevant in males and masculinized hermaphrodites in which the

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ceh-30 gene is relieved of TRA-1-dependent transcriptional repression (Figure 5). How UNC-86, LRS-1, and UNC-132 promote *ced-3* transcription in the CEMs in hermaphrodites remains to be determined.

Why do the CEMs survive for 150 min?

One obvious question that arises from our studies is why CEMs survive for ~150 rather than ~30 min before they undergo apoptosis. The CEMs are born ~320 min after the first cell division and die in hermaphrodites at ~470 min (12). The TRA-1 protein plays a pivotal role in the life-versus-death decision of the CEMs. In the CEMs, TRA-1 represses *ceh-30* transcription thereby causing *egl-1* and *ced-3* de-repression and apoptosis induction. The activity of TRA-1 is regulated at the post-translational level by sex-specific cleavage and proteolysis (37, 38). Specifically, in males, TRA-1 is subject to proteasomal degradation. In hermaphrodites, however, TRA-1 is processed and phosphorylated, which generates a stable and active TRA-1 fragment capable of controlling transcription. Currently it is unclear when during embryonic development active TRA-1 protein is first generated in hermaphrodites. However, experiments using a temperature-sensitive loss-of-function mutation of the gene *tra-2*, which acts upstream of *tra-1* in somatic sex determination, suggest that active TRA-1 might be generated in hermaphrodites starting at ~320 min after the first cell division (39). This notion is supported by the finding that the *ceh-30* gene, whose expression is directly repressed by TRA-1, is no longer expressed at ~360 min (Figure 5, Figure 7). Based on these observations, we propose that the CEMs die only at ~470 min, because a level of active TRA-1 first has to be generated in the CEMs in hermaphrodites that is sufficient for the transcriptional repression of *ceh-30*, which is a prerequisite for the transcriptional de-repression of *egl-1* and *ced-3* transcription at ~390 and 460 min, respectively, and, hence, apoptosis induction (Figure 7). Hence, we propose that the timing of the CEM death is controlled by the sex determination pathway and, in particular, its terminal, global regulator, the Gli-like transcription factor TRA-1.

Potential role for the co-expression of BH3-only and caspase genes in the developmental control of apoptosis

At least during animal development, pro-caspases are thought to be present in most if not all cells (2–5). Furthermore, pro-caspases are thought to be present at levels that, once they become matured and activated, are sufficient for apoptosis induction. Since the maturation and activation of pro-caspases generally represents the commitment of a cell to the apoptotic fate, it is a highly regulated process (40, 41). In contrast, very little is known thus far about the contribution of transcriptional control in the regulation of pro-caspases. The most comprehensive studies available to date were performed in *D. melanogaster*. These studies suggest that transcriptional regulation of caspase genes plays an important role in apoptosis induction at least during *D. melanogaster* development (42). Specifically, it was found that ecdysone-induced apoptosis during Drosophila metamorphosis is dependent on the transcriptional upregulation of the caspase genes *DRONC* and *DRICE* (43–45). The data presented here demonstrate that the transcriptional up-regulation of the *C. elegans* caspase gene *ced-3* is required for the sexually-dimorphic, apoptotic death of the CEM neurons. Based on these findings, we propose that the transcriptional up-regulation of caspase genes is a conserved aspect of apoptosis induction during animal development. Furthermore, our

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data demonstrate that in *C. elegans*, genes encoding BH3-only proteins and pro-caspases can be under the same transcriptional control. We hypothesize that such co-regulatory mechanisms have evolved to ensure efficient apoptosis induction during development.

Materials and Methods

Strains and general methods

C. elegans strains were cultured as described (46). Bristol N2 was used as the wild-type strain. N2 and the CB4856 (Hawaii) strain were used for SNP mapping. Mutations and integrated transgenes used in this study are listed below and are described (47) except where noted otherwise: LG II: *rrf-3(pk1426*) (48). LG III: *ced-4(n1162), ced-3(n717), unc-86(bc151)* (this study)*, lrs-1(bc155)* (this study)*, dpy-17(e164), unc-93(e1500*sd). LG V: *bcIs37* (P_{egl-1}*his-24::gfp*) (16), *egl-1(n1084 n3082)* (13), *sel-10(n1077), unc-132(bc159)* (this study)*, unc-76(e911), him-5(e1490), him-5(e1467ts), dpy-11(e224).* LG X: *ceh-30*(*bc272*) (P. Grote and B. Conradt; unpublished data), *lin-15(n765ts), bcIs9* (P_{pkd-2}*gfp*) (49). The following extrachromosomal arrays were used: *nEx1171* (P_{ceh-30}*ceh-30::gfp*) (22), *bgEx21* (P_{unc-53}*gfp*) (34), *syEx301* (P_{lov-1}*gfp*) (26), *nsEx37* (P_{cfi-1}*gfp*) (33)*, akEx32* (P_{glr-4}*glr-4::gfp*) (32).

RNAi by feeding was performed as described using 6 mM IPTG (50). The plasmids pBC966 (*W08A12.1b(RNAi)*) and pBC967 (*W08A12.1c(RNAi)*) contained full-length cDNA clones cloned as NcoI-NheI fragments into vector L4440. *sel-10(n1077); bcIs9* animals were mutagenized with EMS (ethyl-methanesulfonate) as described (46). Germline transformations were performed as described (51). Cosmids and fosmids were injected at a concentration of 10 ng/μl using pRF4 (*rol-6(su1006)*) at 50 ng/μl as coinjection marker. The plasmid P_{ced-3}*gfp* was injected at 40 ng/ul using pRF4 (*rol-6(su1006)*) (50 ng/μl) as coinjection marker. In the case of *bcIs9* (P_{pkd-2}*gfp*), *syEx301* (P_{lov-1}*gfp*), *bgEx21* (P_{unc-53}*gfp*), *nsEx37* (P*cfi-1gfp*), *akEx32* (P_{glr-4}*glr-4::gfp*), *bcIs37* (P_{egl-1}*his-24::gfp*) and *nEx1171* (P_{ceh-30}*ceh-30::gfp*), transgenic animals were crossed with *unc-86(bc151), lrs-1(bc155)* or *unc-132(bc159)* mutants to generate *unc-86(bc151), lrs-1(bc155)* and *unc-132(bc159)* strains carrying these arrays. pBC957 (P_{ced-3}*gfp*) was constructed by removing an internal, 4.6 kb EcoRV fragment containing most of the *ced-3* coding sequence from a *ced-3* reporter (10).

Cloning of *unc-86(bc151), lrs-1(bc155)* and *unc-132(bc159)*

Standard genetic techniques were used to map *bc151* to the right of *dpy-17* on LG III, *bc155* between *unc-93* and *dpy-17* on LG III, and *bc159* to the left of *dpy-11* on LG V.

bc151: *bc151* failed to complement *unc-86(n846)*, a lf allele of the gene *unc-86*, indicating that *bc151* is a loss-of-function mutation in the *unc-86* gene.

bc155: SNP mapping was used to locate *bc155* between the SNPs C30D11:9408 and R10E4:10469. The fosmid *WRM0638-J8* rescued the phenotype observed in *bc155; sel-10(n1077); bcIs9* animals.

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bc159: SNP mapping was used to map *bc159* to the left of SNP H10D18:26865. The cosmid *W08A12* rescued the phenotype observed in *bc159; sel-10(n1077); bcIs9* animals. The *bc159* phenotype was also rescued by a 31.3 kb XcmI subclone of *W08A12* (pBC430, containing the genes *W08A12.1, W08A12.2* and *egr-1*) and was partially rescued by a 23.9 kb ApaI subclone of *W08A12* (pBC431, spanning the genes *W08A12.4*, *egr-1*, and *W08A12.1*).

Phenotypic analysis

The presence of CEMs in adults was analyzed using *bcIs9* (P_{pkd-2}*gfp*) and a Zeiss Axioskop2 equipped with epifluorescence as described (49). The α GFP-positive CEMs was calculated by dividing the number of CEMs observed by the maximum number of possible CEMs (four CEMs per animal). The presence of CEMs in L4 larvae was analyzed using DIC as described (17). The presence of CEMs in embryos was analyzed as follows. CEMs were identified by DIC in 1½ -fold stage embryos (~450 min) based on their positions and observed for at least ~30 min. (Since the positions of the two dorsal CEMs are less characteristic, these cells could not always be examined.) CEM corpses appear in 2-fold embryos (~465 min). Microscopy of living embryos was performed by mounting embryos on 2% agar pads in M9 buffer, using a Zeiss Axioskop2 equipped with epifluorescence, a Micromax CCD camera (Princeton Instruments), and Metamorph software. DIC and epifluorescence images were taken every 5 min between the 1½- and 2-fold stage to determine the fate of the CEMs and the expression of the GFP reporters in embryonic CEMs.

The presence of the AMso (amphid socket) cells was scored in adults using P_{unc-53}*gfp* as described (32). The presence of the URA cells was determined in adults using two different reporters, P_{glr-4}*grl-4::gfp* and P_{cfi-1}*gfp*, as described (33).

4-D microscopy and lineage analysis

4-D microscopy and lineaging analysis was performed on *C. elegans* embryos as previously described (52, 53). Embryos were recorded at 20°C, and GFP expression of the reporters P_{ceh-30}*ceh-30::gfp*, P_{egl-1}*his-24::gfp* and P_{ced-3}*gfp* was recorded after every 30 DIC stacks. The 4-D recordings were analyzed using the SIMI BioCell software as previously described (52, 54). The ventral left CEM (CEMVL, ABplpaapapp) was lineaged in all embryos. Starting at the 2 to 4-cell stage, cells were tracked and their 3-D coordinates were saved approximately every 2 min, until at least ~465 min after the first cell division. Additionally, the 3-D coordinates of the CEMVL progenitor (ABplpaapap) or CEMVL were saved at the time points when GFP expression of the reporters P_{ceh-30}*ceh-30::gfp*, P_{egl-1}*his-24::gfp* or P_{ced-3}*gfp* was recorded. Using this approach, the status of expression of these reporters in the CEM could be determined. The cell death fate of the CEMVL was determined at ~465 min by DIC.

Supplementary Material

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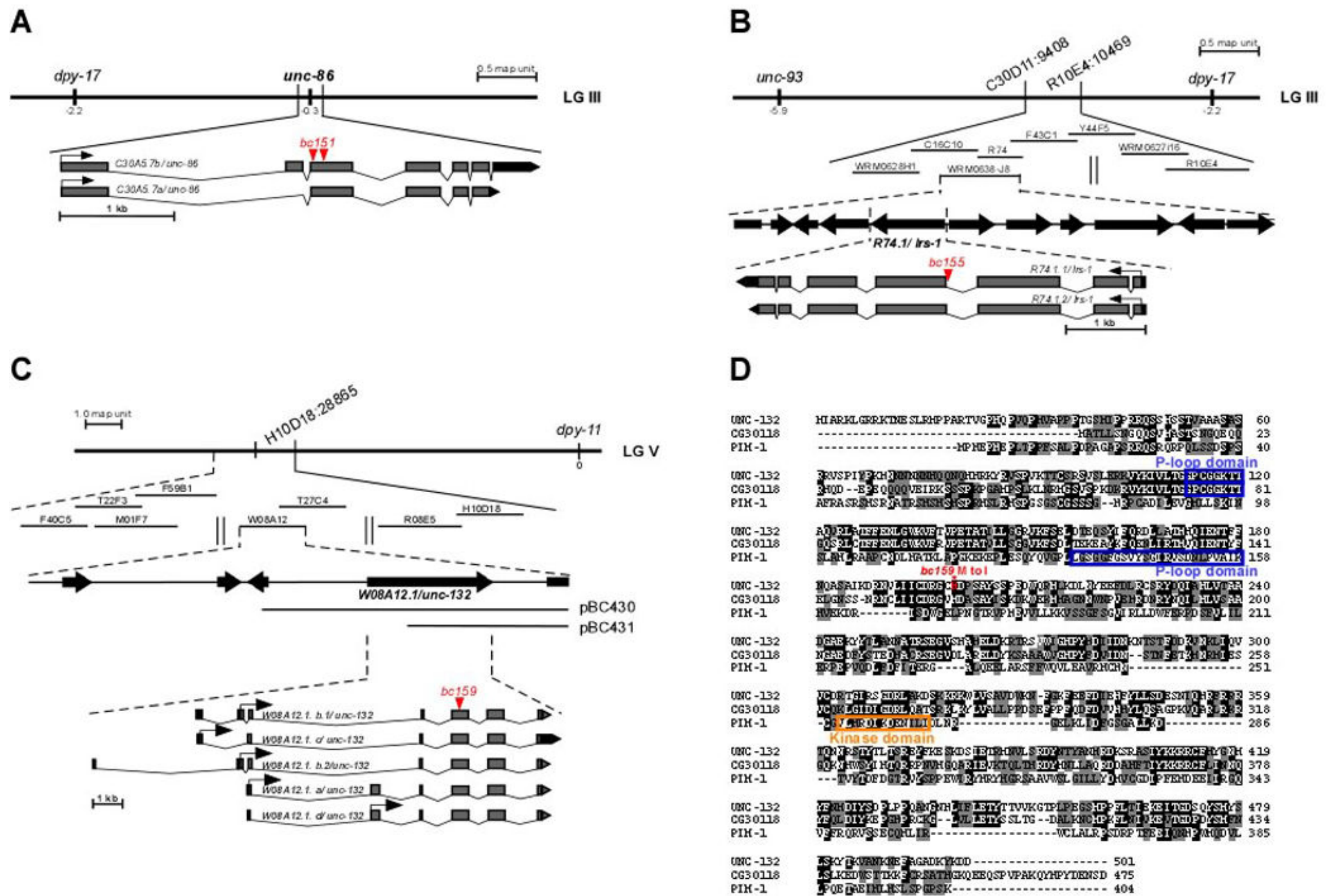

Fig. 1. Cloning of *unc-86(bc151)*, *lrs-1(bc155)* and *unc-132(bc159)*

(A) The gene *dpy-17* used for mapping *bc151* is indicated on the genetic map (LG III). The two mutations identified in the *C30A5.7/unc-86* gene in *bc151* animals are indicated in red. **(B)** Genes and SNPs used for mapping *bc155* on LG III are indicated on the genetic map. Cosmids and fosmids tested for *bc155* rescue are shown below the genetic map. The *bc155* mutation in *R74.1/lrs-1* is indicated in red. **(C)** *dpy-11* and H10D18:28865 used for mapping *bc159* on LG V are indicated on the genetic map. Cosmids tested for *bc159* rescue are shown below. pBC430 and pBC431 represent rescuing subclones of cosmid *W08A12*. The *bc159* mutation in *W08A12.1/unc-132* is indicated in red. (D) Alignment of the protein sequences of UNC-132 (isoform a), *D. melanogaster* CG30118 and human PIM-1. The alignment was done using the EMBOSS algorithm. Identical amino acids have a black background. Amino acids with similar biochemical properties have a gray background. The blue boxes indicate the P-loop domains and the orange box the kinase domain. The amino acid that is changed as a result *bc159* (M200I) is indicated by a red asterisk.

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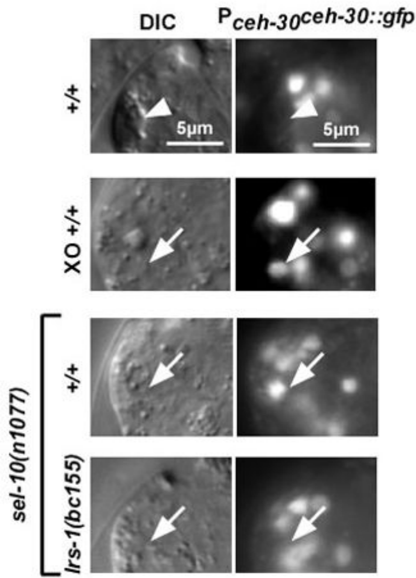

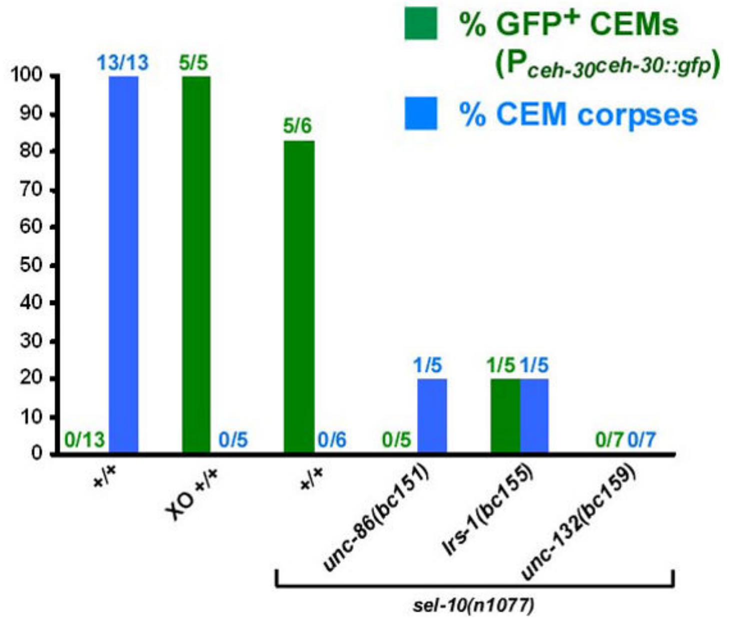

Fig. 2. *unc-86, lrs-1* and *unc-132* are required for *ceh-30* expression in the CEMs in masculinized hermaphrodites

(A) DIC and fluorescence images ($P_{ceh\text{-}30}$*ceh-30::gfp*) of CEM corpses (white arrow heads) or CEMs (white arrows) in wild-type hermaphrodites (+/+), males (XO +/+), *sel-10(n1077)* hermaphrodites or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. **(B)** Summary of data obtained on $P_{ceh\text{-}30}$*ceh-30::gfp* expression. Green bars indicate the percentage of CEMs that were GFP-positive and green numbers above indicate the fraction of CEMs analyzed that were GFP-positive. Blue bars indicate the percentage of CEMs that acquired a corpse-like morphology and blue numbers above indicate the fraction of CEMs analyzed that had a corpse-like morphology. All strains analyzed were homozygous for *unc-76(e911)* and carried the extrachromosomal array *nEx1171* ($P_{ceh\text{-}30}$*ceh-30::gfp*). The wild-type hermaphrodites and males analyzed were homozygous for *him-5(e1467ts)*. The strains *unc-86*(*bc151*); *sel-10(n1077)* and *lrs-1(bc155)*; *sel-10(n1077)* were also homozygous for *dpy-17(e164)*.

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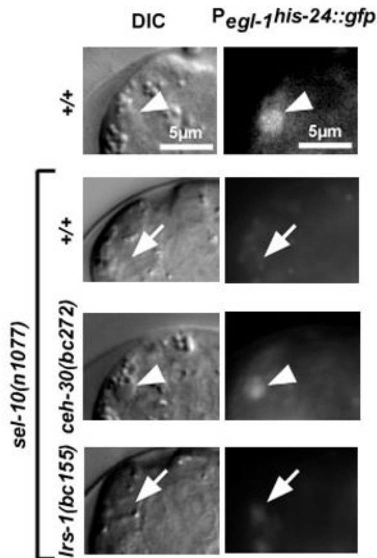

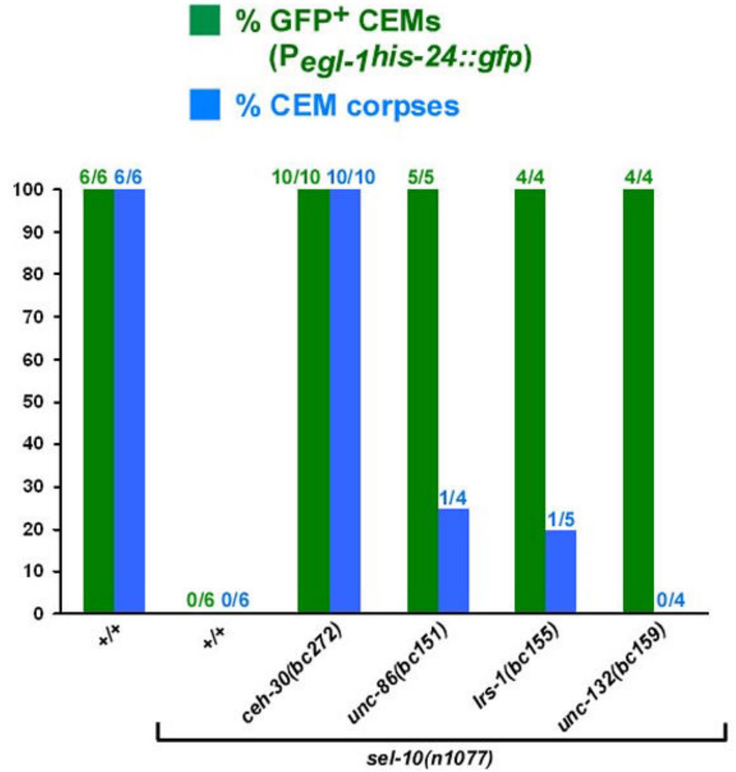

Fig. 3. *egl-1* is transcribed in surviving CEMs in masculinized *unc-86(bc151), lrs-1(bc155),* or *unc-132(bc159)* hermaphrodites

(A) DIC and fluorescence images (P_{egl-1}*his-24::gfp*) of CEMs (white arrows) or CEM corpses (white arrow heads) in wild-type hermaphrodites (+/+), *sel-10(n1077)*, *sel-10(n1077); ceh-30(bc272)* or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. **(B)** Summary of data obtained on P_{egl-1}*his-24::gfp* expression. Green bars indicate the percentage of CEMs that were GFP-positive and blue bars indicate the percentage of CEMs that acquired a corpse-like morphology. All strains analyzed were homozygous for the integrated P_{egl-1}*his-24::gfp* array *bcIs37*. The strain *ceh-30(bc272); sel-10(n1077)* was also homozygous for the integrated array *bcIs9*. The strains *unc-86*(*bc151*); *sel-10(n1077)* and *lrs-1(bc155)*; *sel-10(n1077)* were homozygous for *dpy-17(e164)*.

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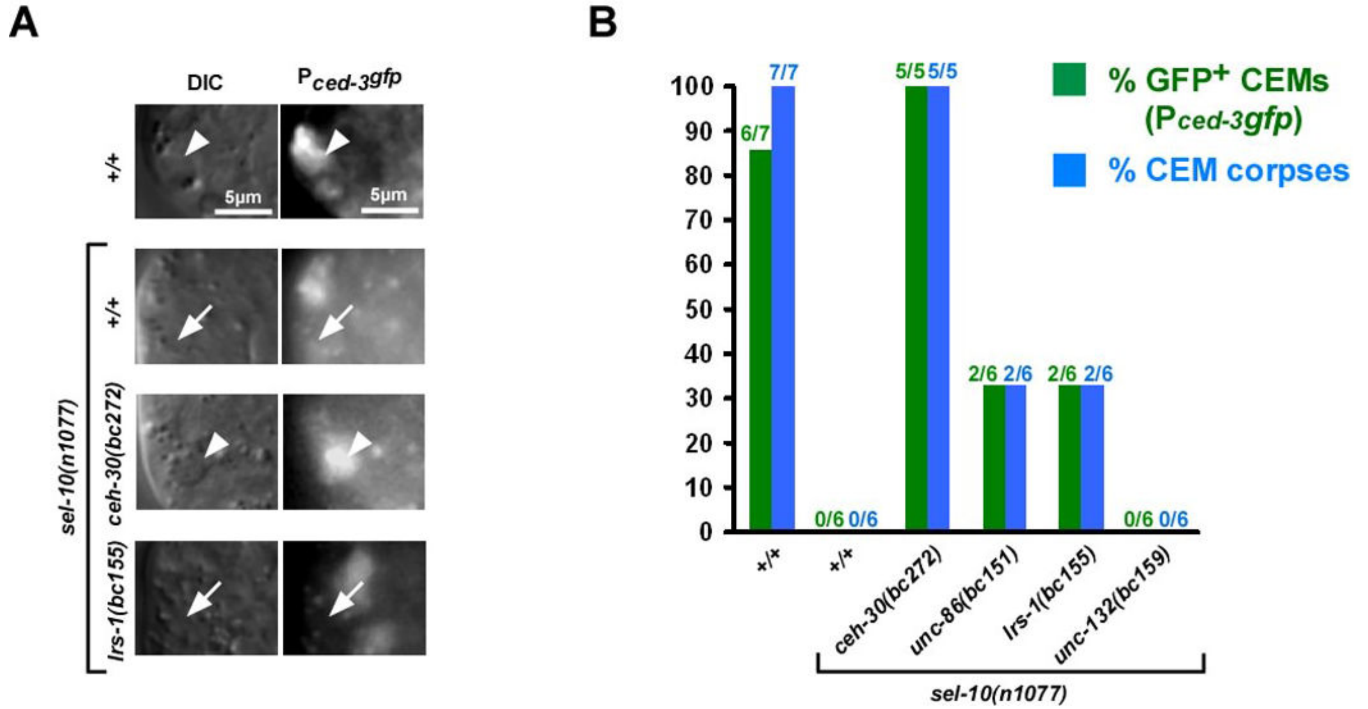

Fig. 4. Analysis of *ced-3* transcription in the CEMs

(A) DIC and fluorescence images ($P_{ced\text{-}3}gfp$) of CEM corpses (white arrow heads) or CEMs (white arrows) in wild-type (+/+), *sel-10(n1077)*, *sel-10(n1077); ceh-30(bc272)* or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. **(B)** Summary of data obtained on $P_{ced\text{-}3}gfp$ expression. Green bars indicate the percentage of CEMs that were GFP-positive and blue bars indicate the percentage of CEMs that acquired a corpse-like morphology. All strains analyzed were homozygous for the integrated $P_{pkd\text{-}2}gfp$ array *bcIs9* and carried extrachromosomal arrays of $P_{ced\text{-}3}gfp$ (+/+ [*bcEx834*, *bcEX836*], *sel-10(n1077)* [*bcEx839*, *bcEx840*], *ceh-30(bc272)*; *sel-10(n1077)* [*bcEx876*], *unc-86(bc151); sel-10(n1077)* [*bcEx870*, *bcEx871*], *lrs-1(bc155); sel-10(n1077)* [*bcEx872*, *bcEx873*], *unc-132(bc159)*; *sel-10(n1077)* [*bcEx874*, *bcEx875*]).

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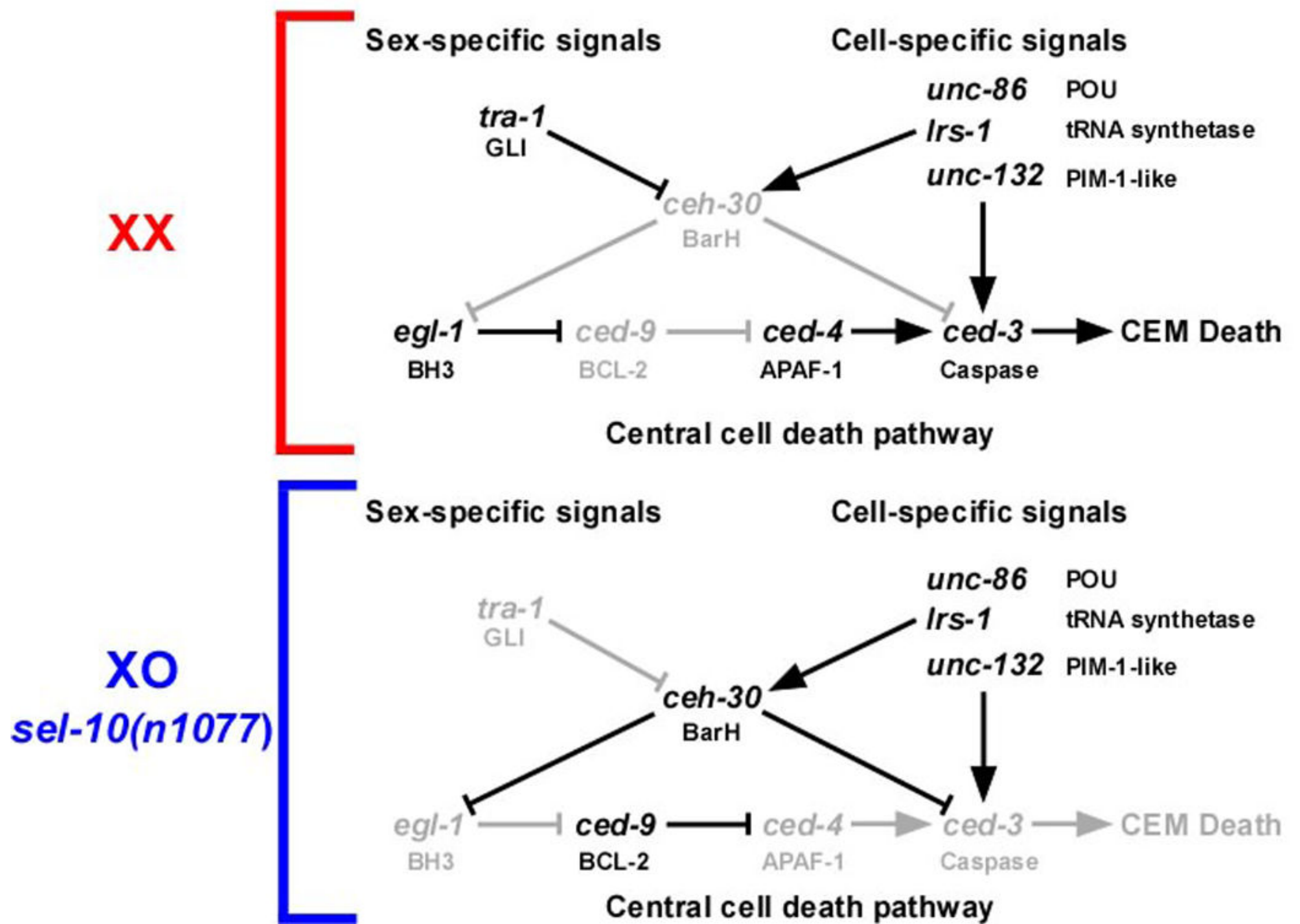

Fig. 5.

Genetic pathway of the CEM death. Upper panel. In wild-type hermaphrodites (XX), *tra-1* is active in the CEMs, thereby blocking *ceh-30*. Thus the pro-apoptotic genes *egl-1* and *ced-3* are active, which promotes CEM death. See text for details. Lower panel. In males (XO) or masculinized hermaphrodites (*sel-10*(*n1077*)), *tra-1* is not active in the CEMs, which results in the activation of *ceh-30*, and, consequently, the repression of *egl-1* and *ced-3*. Thus, CEM death is inhibited. See text for details.

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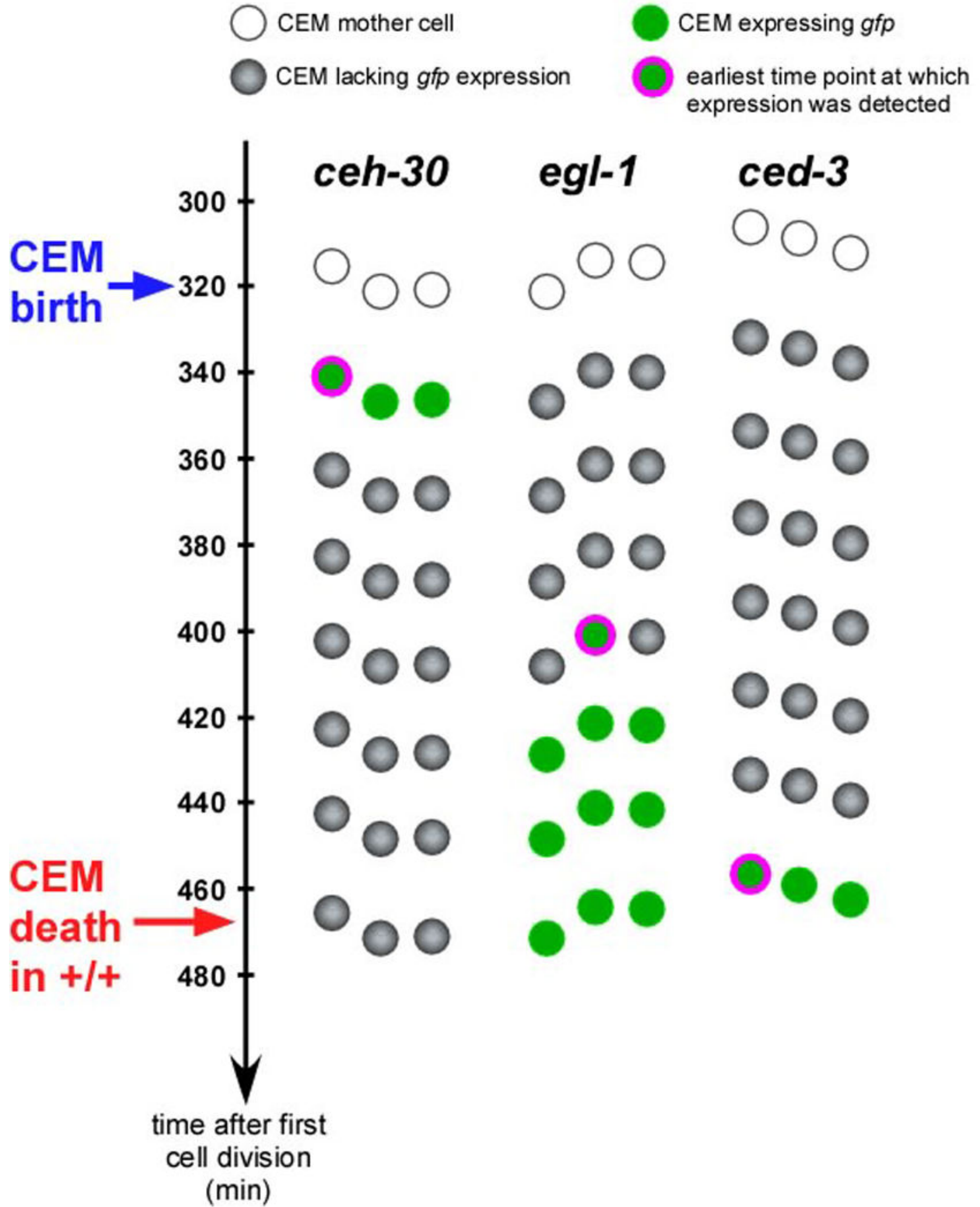

Fig. 6. *ceh-30, egl-1* and *ced-3* expression in embryonic CEMs in wild-type hermaphrodites

Schematic representation of time course analyses of the expression of the reporters $P_{ceh\text{-}30}$*ceh-30::gfp*, $P_{egl\text{-}1}$*his-24::gfp*, and $P_{ced\text{-}3}$*gfp* in the ventral left embryonic CEM (CEMVL) in wild-type hermaphrodites. Time (min) is indicated on the left. Three embryos were analyzed. White dots represent the CEM mother cells, green dots indicate CEMs expressing a particular *gfp* reporter. Green dots surrounded by purple circles indicate CEMs at the time when expression of the *gfp* reporter was first detected. (See Figure S1 for corresponding DIC and fluorescent images.) Gray dots indicate CEMs lacking reporter

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expression. Embryos were prepared for 4-D microscopy and lineaged starting at the 2- or 4-cell stage as described in Materials and Methods. Fluorescent stacks were taken every ~20 min after the CEM mother cell was born. No expression was detected for any of the reporters in the CEM mother cell.

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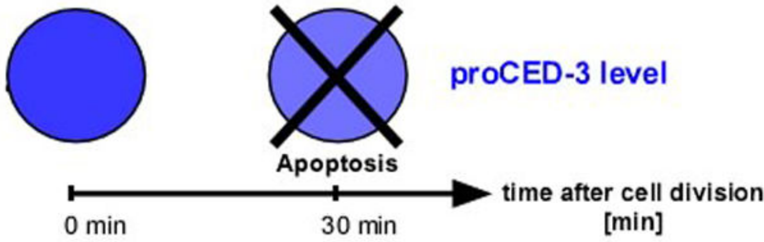

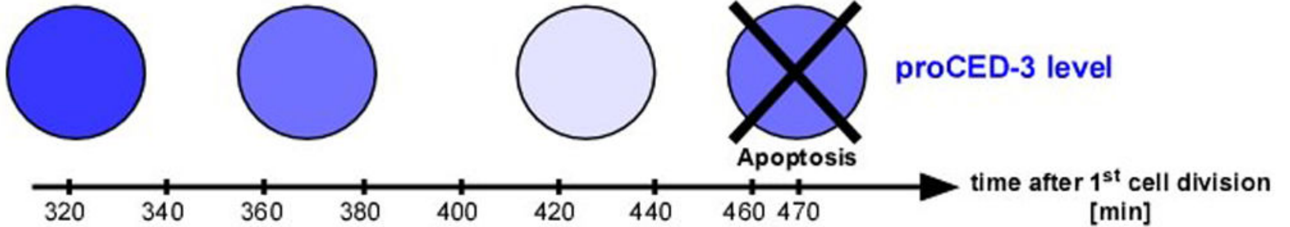

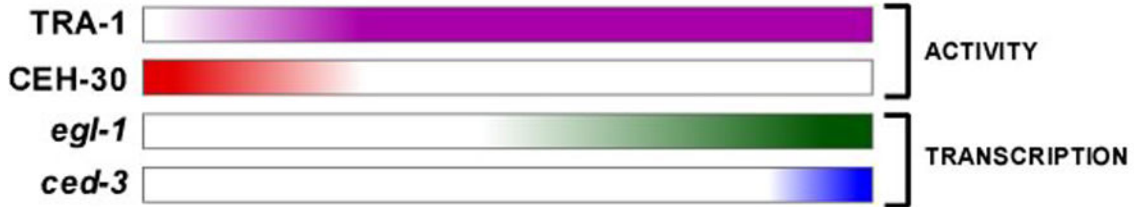

Fig. 7. Model of the life-versus-death decision in the CEMs

Top panel. Most cells programmed to die do so ~30 min after being generated. In these cells, proCED-3 protein inherited from the progenitor is sufficient for apoptosis induction in response to *egl-1* transcriptional activation. Bottom panel. The CEMs in hermaphrodites die ~150 min after being generated (~470 min after the first cell division). Increasing TRA-1 activity results in decreasing CEH-30 activity, which is required for the de-repression of *egl-1* and *ced-3* transcription. The activation at the transcriptional level of the *ced-3* gene compensates for decreased levels of proCED-3. See text for details.

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Table 1

The CEMs are absent in masculinized hermaphrodites or males homozygous for *unc-86(bc151), lrs-1(bc155)* or *unc-132(bc159)*.

A. Presence of differentiated CEMs in adults

Genotype	% GFP+ CEMs (n)							
	+/+				***sel-10(n1077)***			
					+/+		***ced-3(n717)***	
	XX		**XO**[b]		**XX**		**XX**	
+/+	0	(many)	100	(many)	100	(many)	100	(many)
egl-1(n1084 n3082)	68	(356)		ND		ND		ND
ced-3(n717)	78	(232)		ND		ND		ND
unc-86(bc151)[a]		ND	25	(80)	12	(178)	8	(92)
lrs-1(bc155)[a]		ND	18	(76)	10	(224)	7	(80)
unc-132(bc159)		ND	0	(68)	0	(232)	0	(96)

B. Presence of CEMs in L4 larvae

Genotype	% CEMs (n)					
	+/+		***sel-10(n1077)***		***ced-3(n717); sel-10(n1077)***	
	XX		**XX**		**XX**	
+/+	0	(36)	100	(40)	100	(36)
egl-1(n1084 n3082)	96	(80)		ND		ND
ced-3(n717)	98	(128)		ND		ND
unc-86(bc151)[a]		ND	6	(32)	4	(24)
lrs-1(bc155)[a]		ND	7	(48)	7	(48)
unc-132(bc159)		ND	0	(20)	2	(44)

The presence of differentiated CEMs was analyzed in adults using the $P_{pkd\text{-}2}$ *gfp* reporter as described in Materials and Methods. All strains were homozygous for the $P_{pkd\text{-}2}$ *gfp* integration *bcIs9*.

[a]Indicates that the strains were homozygous for *dpy-17(e164)* III.

[b]Indicates that the strains were homozygous for *him-5(e1490)* V.

The presence of CEMs was scored at the L4 stage using DIC as described in Materials and Methods. All strains were homozygous for the $P_{pkd\text{-}2}$ *gfp* integration *bcIs9*.

[a]Indicates that the strains were homozygous for *dpy-17(e164)* III.

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Table 2

The inactivation by RNAi of *lrs-1* and other genes encoding tRNA synthetases as well as *unc-132* by RNAi results in the absence of differentiated CEMs in masculinized hermaphrodites.

A. Inactivation by RNAi of *lrs-1* and other genes encoding tRNA synthetases

Genotype	tRNA synthetase affected	%GFP+ CEMs (n) *sel-10(n1077)* XX		Other RNAi phenotypes
+/+	NA	100	(many)	NA
Class I tRNA synthetases				
lrs-1(RNAi)	Leu	**62**	(60)	larval arrest, lethality
irs-2(RNAi)	Ile	**63**	(248)	none detected
irs-1(RNAi)	Ile	83	(12)	larval arrest, lethality
vrs-2(RNAi)	Val	**60**	(32)	larval arrest, lethality
vrs-1(RNAi)	Val	90	(124)	none detected
mrs-1(RNAi)	Met	89	(132)	none detected
Class II tRNA synthetases				
hrs-1(RNAi)	His	**71**	(24)	larval arrest, lethality
srs-1(RNAi)	Ser	95	(112)	none detected
yrs-1(RNAi)	Tyr	88	(68)	none detected
ers-1(RNAi)	Glu	85	(100)	none detected
wrs-2(RNAi)	Trp	**75**	(124)	none detected
drs-1(RNAi)	Asp	92	(24)	larval arrest, lethality
prs-1(RNAi)	Pro	97	(116)	none detected
ars-1(RNAi)	Ala	88	(124)	none detected
trs-1(RNAi)	Thr	**74**	(72)	none detected
nrs-1(RNAi)	Asn	86	(28)	none detected
frs-1(RNAi)	Phe	100	(36)	larval arrest, lethality
crs-1(RNAi)	Cys	91	(108)	larval arrest, lethality
krs-1(RNAi)	Lys	100	(32)	larval arrest, lethality

B. Inactivation of *unc-132* by RNAi

Genotype	% GFP+ CEMs (n) *sel-10(n1077)*	
+/+	99	(200)
unc-132(RNAi ; W08A12.1b cDNA*)*	49	(216)
unc-132(RNAi ; W08A12.1c cDNA*)*	64	(172)

The presence of differentiated CEMs was analyzed in adults using the $P_{pkd\text{-}2gfp}$ reporter as described in Materials and Methods. All strains were homozygous for the $P_{pkd\text{-}2gfp}$ integration *bcIs9*. RNAi was performed by feeding as described in Materials and Methods.

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Table 3

The fate of the AMso and URAs in *unc-86(bc151), lrs-1(bc155)* and *unc-132(bc159)*.

Genotype	% GFP+ AMso (n) P_{unc-53}*gfp*		% GFP+ URAs (n) P_{glr-4}*grl-4::gfp*		% GFP+ URAs (n) P_{cfi-1}*gfp*	
+/+	100	(120)	95	(92)	91	(124)
unc-86(bc151) [a]	100	(100)	8	(40)	0	(100)
lrs-1(bc155) [a]	100	(140)	93	(84)	89	(60)
unc-132(bc159)	100	(140)	77	(88)	93	(60)

The presence of the AMso and URAs was determined in hermaphrodites using P_{unc-53}*gfp*(*bgEx21,* AMso) or P_{glr-4}*grl-4::gfp* (*akEx32*) and P_{cfi-1}*gfp* (*nsEx37*) (URAs) as described in Materials and Methods. Strains carrying the array *akEx32* were homozygous for *lin-15*(*n765*ts) X. Strains carrying the array *nsEx37* were homozygous for *him-5(e1490)* V and *lin-15(n765*ts*)* X.

[a]Indicates that the strains were homozygous for *dpy-17(e164)* III.